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Delayed Injection of a Physically Cross-Linked PNIPAAm-*g*-PEG Hydrogel in Rat Contused Spinal Cord Improves Functional Recovery

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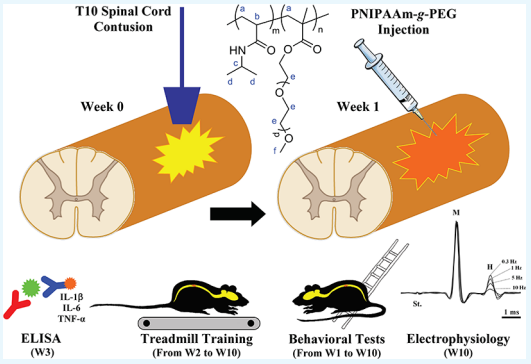
ABSTRACT: Spinal cord injury is a main health issue, leading to multiple functional deficits with major consequences such as motor and sensitive impairment below the lesion. To date, all repair strategies remain ineffective. In line with the experiments showing that implanted hydrogels, immunologically inert biomaterials, from natural or synthetic origins, are promising tools and in order to reduce functional deficits, to increase locomotor recovery, and to reduce spasticity, we injected into the lesion area, 1 week after a severe T10 spinal cord contusion, a thermoresponsive physically cross-linked poly(*N*-isopropylacrylamide)-poly(ethylene glycol) copolymer hydrogel. The effect of postinjury intensive rehabilitation training was also studied. A group of male Sprague−Dawley rats receiving the hydrogel was enrolled in an 8 week program of physical activity (15 min/day, 5 days/week) in order to verify if the combination of a treadmill step-training and hydrogel could lead to better outcomes. The data obtained were compared to those obtained in animals with a spinal lesion alone receiving a saline injection with or without performing the same program of physical activity. Furthermore, in order to verify the biocompatibility of our designed biomaterial, an inflammatory reaction (interleukin-1β, interleukin-6, and tumor necrosis factor-α) was examined 15 days post-hydrogel injection. Functional recovery (postural and locomotor activities and sensorimotor coordination) was assessed from the day of injection, once a week, for 9 weeks. Finally, 9 weeks postinjection, the spinal reflexivity (rate-dependent depression of the *H*-reflex) was measured. The results indicate that the hydrogel did not induce an additional inflammation. Furthermore, we observed the same significant locomotor improvements in hydrogel-injected animals as in trained saline-injected animals. However, the combination of hydrogel with exercise did not show higher recovery compared to that evaluated by the two strategies independently. Finally, the *H*-reflex depression recovery was found to be induced by the hydrogel and, albeit to a lesser degree, exercise. However, no recovery was observed when the two strategies were combined. Our results highlight the effectiveness of our copolymer and its high therapeutic potential to preserve/repair the spinal cord after lesion.



1. INTRODUCTION

Traumatic injuries of the spinal cord cause devastating and irreversible losses of function. These injuries cause tissue damage and disrupt the internal intricate circuits of the spinal cord and its external connections that are involved in sensorimotor and autonomic functions. The primary insult initiates a complex secondary injury cascade of events leading to supplementary cell death, ischemia, inflammation, formation of a glial scar, and cystic cavities.[1,2] Associated with the poor intrinsic recovery potential of the adult spinal cord, these changes in the organization and structural architecture cause permanent neurological deficits.

After the spinal cord injury (SCI), the course of recovery is highly variable. This recovery depends on the level (cervical, thoracic, lumbar, and sacral) of the lesion, the degree of tissue loss (leading to neuronal death and cell dysfunction in the area of the lesion and in the remote spinal cord connected to the area of tissue damage) and preservation, the immediate cares (pharmacological protection, stabilization, and decompression of the spinal cord) limiting the extension of the injury, the chronic management of the patient (physical rehabilitation and functional electrical stimulation) and experience-driven relearning, and the plasticity of the spinal cord (change of properties of existing neuronal pathways, formation of new

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connections, dendritic arborization remodeling, and axonal sprouting), which depends on several factors such as age, life history, and motivation.[1−3] Thus, recovery is dynamic and multifactorial but remains limited in adult mammals and humans. It depends on internal factors specific to the species itself and external factors such as post-traumatic immediate intervention and intensive rehabilitation.

Since the discovery that damaged axons could grow in the central nervous system (CNS) of an adult mammal,[4] a number of strategies have been developed to limit the deficits and promote recovery after a SCI. Among them, exercise training has been proved to (1) improve the function of the skeletal muscle through reshaping its structure and muscle fiber type, (2) regulate the physiological and metabolic functions of a motoneuron, and (3) remodel the function of cerebral cortex.[5] Physical exercise is known to increase the brain-derived neurotrophic factor (BDNF) expression and axonal regeneration and reduce cavity formation.[6−11]

Biomaterials are nonpharmacological emerging therapies that present several advantages for spinal cord repair because of their structural and chemical versatility.[12] Furthermore, the use of biomaterials is an exciting strategy to fill the postinjury cystic cavities and to support for cell migration and axon growth by reproducing the complex structural architecture of the extracellular matrix.[13−15] Among these biomaterials, synthetic hydrogels have been shown to be effective in the animal model of SCI.[16−19] More recently, great interest has been found in "smart or intelligent" hydrogels responding, for example, to stimuli, such as pH, light radiation, temperature, magnetic and electric field, ionic concentration, and ultrasound.[20,21] These hydrogels are in liquid form before the stimulus is applied, and after stimulation, they changed into a gel. Thus, when injected in the spinal lesion, these hydrogels can fill the irregular and/or multishape cavities and be more efficient than the implanted hydrogels.[22]

Poly(*N*-isopropylacrylamide) (PNIPAAm)-based thermoresponsive hydrogels are highly attractive because of suitable lower critical solution temperature (LCST, ∼32 °C) between the room and physiological temperature, making them easily handled and injectable at room temperature (liquid state) and fast-gelling upon heating to 37 °C. Yet, the significant hydrophobicity of PNIPAAm above LCST requires adjunction of hydrophilic moieties, such as polyethylene glycol (PEG), to ensure water retention and prevent gel shrinkage.[23] In this context, a chemically (use of PEG-dimethacrylate) cross-linked PNIPAAm-*g*-PEG hydrogel is a thermoresponsive hydrogel that has been used to repair the spinal cord.[23−26] When combined with BDNF or NT-3, this hydrogel (1) exhibits biocompatibility with mesenchymal stem cells, (2) has a compressive modulus close to the spinal cord tissue, (3) allows delivery of skin fibroblast transplants, (4) is permissive to axonal regeneration, and (5) sustains a drug release for up to 4 weeks, showing it great therapeutical potential for SCI.

However, because of permanent cross-links, chemically cross-linked hydrogels are nonhomogeneous and more viscous at room temperature (i.e., below LCST) and less easy to handle than physically cross-linked thermoresponsive hydrogels which are more appropriate for the incorporation of bioactive substances.[27] Furthermore, difficult renal clearance of chemically cross-linked hydrogels (as permanent cross-links make it impossible to recover individual polymer chains) is a major drawback, making them potentially toxic in long-term accumulation.[28]

In addition, as nondegradable polymers, hydrogel calcification may induce inflammatory response that might limit long-term axonal regeneration when injected into a lesion cavity after SCI.[29,30] Finally, axonal regrowth is restricted to the existing pores of the biomaterial. Indeed, because the axons of the spinal cord have a diameter ranging from 0.1 to 6 μm, with more than 90% of axons smaller than 1.5 μm, pores smaller than 0.1 μm will not be colonized by regrowing axons.

Thus, if each of these cross-linked hydrogels presents advantages and drawbacks, chemically cross-linked hydrogels seem to be more suitable for local application (i.e., gel and patch form for transdermal drug delivery) and physically cross-linked hydrogels for internal application (i.e., injection into the spinal cord).

In the present study, we injected, 1 week postinjury, a thermoresponsive and thermoreversible physically cross-linked PNIPAAm-*g*-PEG hydrogel into a T10 contused spinal cord in order to fill the lesion cavities that formed during the secondary lesion. In order to increase the recovery, a group of treated animals was made to perform a daily treadmill exercise for 8 weeks postinjection. The treated rats were compared to animals that received a saline solution with or without performing a treadmill exercise. We hypothesized that a combination of a promising emerging therapy and an intensive rehabilitation program could lead to higher sensorimotor recovery in a model of thoracic spinal cord contusion and delayed injection of a thermosensitive and thermoreversible PNIPAAm-*g*-PEG copolymer. Inflammatory reaction was evaluated 2 weeks postinjection. Then, sensorimotor recovery was evaluated from the day of injection (W1, 1 week postinjury) to the ninth (W10) week postinjection. Finally, electrophysiological recordings allowed determining the functional state of the sensorimotor loop below the lesion.

2. MATERIALS AND METHODS

2.1. Animals. Experiments were performed on 48 adult male Sprague Dawley rats weighing between 250 and 300g (Élevage JANVIER, Centre d'Elevage Roger JANVIER, Le Genest Saint Isle, France) housed two per cage in smooth-bottomed plastic cages in a colony room maintained on a 12:12 h light/dark photoperiod and at 22 °C. Food (rat chow, Safe, Augy, France) and drinking water were made available ad libitum. The health status of the animals was controlled on a daily basis, and animals were housed in the animal facility for 2 weeks before the initiation of the experiment (recording of the PRE-values).

2.2. Ethical Considerations. Experiment was performed according to the French law (Decrees and orders N°2013-118 of 01 February 2013, JORF n°0032) on animal care guidelines and after approval by animal Care Committees of Aix-Marseille Université and Centre National de la Recherche Scientifique. All individuals conducting the research were listed in the authorized personnel section of the animal research protocol (License n°A13.013.06). Furthermore, experiments were performed following the recommendations provided in the Guide for Care and Use of Laboratory Animals (U.S. Department of Health and Human Services, National Institutes of Health) and in accordance with the directives 86/609/EEC and 010/63/EU of the European Parliament and of the Council of 24 November 1986 and of 22 September 2010, respectively, and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. Animals presenting the sign

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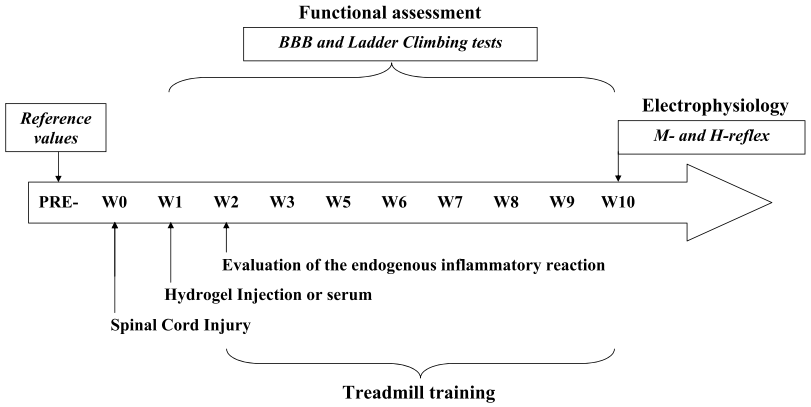

Figure 1. Experimental design. The week before surgery, the reference values (PRE-) of each functional test were recorded. Surgery was performed at W0. Then, for 9 weeks (1 week postinjury, W1−W10), the tests were performed, once a week. Hydrogel or serum was injected at W1. Endogenous inflammatory reaction was analyzed at W3 (2 weeks postinjection or 3 weeks postinjury). Animals involved in the exercise protocol were trained 5 days/week from W2 to W10. At W10, electrophysiological examination allowed to record M and *H*-waves.

of suffering such as screech, prostration, hyperactivity, anorexia, and paw-eating behavior were sacrificed.

2.3. Copolymer Preparation and Characterization. Poly(*N*-isopropylacrylamide)-*co*-poly(ethylene glycol)] methacrylate [p(NIPAAm-*co*-PEGMA)], also denoted as PNIPAAm-*g*-PEG, was synthesized by radical copolymerization of *N*-isopropylacrylamide (NIPAAm, Merck KGaA, Lyon, France) with poly(ethylene glycol) methacrylate (PEGMA). PEGMA was first synthesized through the reaction of PEG monomethylether (Me-PEG-OH, Merck KGaA) with methacryloyl chloride (Merck KGaA). In brief, 10 g (5 mmol) of Me-PEG-OH (M_n = 2000 g·mol^{-1}) was allowed to react with 5.2 g (49.7 mmol) of methacyloyl chloride (added dropwise) in 100 mL of dichloromethane in the presence of 7 mL (50.2 mmol) of triethylamine (TEA, Acros Organics—Fisher Scientific SAS, Illkirch, France). After 20 h under stirring at room temperature, dichloromethane was removed under reduced pressure and the crude product was diluted with tetrahydrofuran (VWR International S.A.S, Fontenay-sous-Bois, France). After filtration of the TEA salts, the crude solution was precipitated in 90% diethyl ether and 10% ethanol mixture and dried under vacuum.

For copolymerization, NIPAAm (3.68 g), PEGMA (0.263 g), and azobisisobutyronitrile (AIBN, Merck Sigma Aldrich, Lyon, France) initiator (0.10 g) were dissolved in methanol (30 mL) in a two-neck round-bottom flask fitted with a septum and a reflux condenser, and the solution was degassed for 30 min by argon bubbling. The flask was then heated at 68 °C, and the polymerization was allowed to proceed for 20 h. After reconcentration of the mixture by methanol evaporation under reduced pressure, the copolymer was precipitated twice in diethyl ether and dried under vacuum.

Polymers were characterized by ^{1}H NMR in chloroform-*d* ($CDCl_3$) or in deuterated dimethyl sulfoxide (DMSO-d_6) with a spectrometer (Advance III HD, 400 MHz, Bruker BioSpin Corporation, Billerica, Massachusetts, USA) and by size exclusion chromatography (SEC) in dimethylformamide (DMF) using an integrated gel permeation chromatography (GPC)/SEC system (PL-GPC 120, Agilent Technologies, Polymer Laboratories, Varian SA, Marseille, France), as previously described. The LCST of the copolymer was determined by dynamic light scattering (DLS) analysis, as previously described.[31]

Then, the copolymer was dissolved at 13.7 wt % in phosphate buffer sodium (PBS) and heated to 37 °C, at which the hydrogel quickly formed. The rheological properties of the hydrogel at 37 °C were analyzed with a rheometer (MCR 302, Anton Paar France S.A.S, Les Ulis, France), as previously described.[31]

2.4. Protocol Design and Experimental Groups. After 2 weeks of familiarization (1 h per day, 3 days per week) on an open-field, on an inclined ladder, and on the treadmill at different speeds (14−30 m·min^{-1}, 15 min/session), and after measurement of the reference values (PRE-) of each behavioral test, animals were randomly assigned to the following four groups: (1) saline (n = 16) in which a thoracic T10 contusion was performed (W0), followed by a 1 week (W1) delayed injection of saline into the lesion cavity, (2) saline + E (n = 8) in which a thoracic T10 contusion was performed (W0), followed by a 1 week (W1) delayed injection of saline and then animals were enrolled in a daily exercise (E) protocol on a treadmill for 8 weeks from the second (W2) to the tenth (W10) week, (3) the PNIPAAm-*g*-PEG group (n = 16) in which a thoracic T10 contusion was performed (W0), followed by a 1 week (W1) delayed injection of PNIPAAm-*g*-PEG hydrogel into the lesion cavity, and (4) the PNIPAAm-*g*-PEG + E group (n = 8) in which a thoracic T10 contusion was performed (W0), followed by a 1 week (W1) delayed injection of PNIPAAm-*g*-PEG hydrogel into the lesion cavity and then animals were enrolled in a daily training protocol on a treadmill for 8 weeks from the second (W2) to the tenth (W10) week.

Sixteen animals of the saline (n = 8) and PNIPAAm-*g*-PEG (n = 8) groups were sacrificed 2 (W3) weeks after the injection (3 weeks postinjury) in order to evaluate the endogenous inflammation at the lesion site. For other animals (n = 32), sensory and motor recovery in the posterior legs was measured once a week from 1 week before the injury (PRE-) to the 11 subsequent weeks by using two behavioral tests. Then, at W10, spinal reflexivity was evaluated using electrophysiological recordings of the *H*-reflex below the lesion. The chronological order of our experiences is schematically shown in Figure 1.

2.5. Surgery. All surgeries were interspersed throughout the day (during the light cycle). Animals were anesthetized with 3% isoflurane in oxygen (1 L·min^{-1}) given through a mask integrated in a surgical stereotaxic frame. Surgical procedures were performed in sterile conditions with the aid

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of a dissecting microscope. During surgery, body temperature was maintained at 37 °C using a homeothermic feedback-controlled heating pad (Homeothermic Blanket Systems, Harvard apparatus Sarl, Les Ulis, France). The animal was positioned in ventral decubitus, back was shaved, disinfected (betadine, 5%), and a midline dorsal incision was performed over the C6-T13 spinous processes. The superficial muscles were retracted using retractors to expose the thoracic vertebrae. Then, dorsal thoracic laminectomy was performed to expose the spinal cord without affecting the integrity of the spinal cord. The dura was left intact. Stabilization clamps were placed at the posterior processes of the vertebra T9 and T11 to support the vertebral column during impact.

The spinal cord was contused at the thoracic vertebra T10 using a NYU-MASCIS weight-drop impactor (Model III, New York University—Multicenter Animal SCI Study) equipped with a 10 g rod with a flat circular impact surface of diameter 2.5 mm which detects rod velocity (displacement over time) and impact-induced movement using digital optical potentiometers in order to calculate the impact velocity and compression rate. The impact rod was centered above T10 and slowly lowered until it contacted the dura, which was determined by completion of a circuit that activated a tone. Then, the cord was contused by dropping the rod from a height of 50 mm.[32] The force applied through the impactor was around 300 kdyn. The rod was dropped at a velocity of around 1.3 m/s (impact velocity).

Such thoracic lesion isolates the lumbosacral neuronal network (termed the central pattern generator, CPG), dedicated to hindlimb locomotion, from supraspinal structures, leading to paralysis of the lower body parts.[33] However, the CPG remains functional and it is able to generate an alternate rhythmic activity.[10,33−35]

After injury, muscles were sutured in anatomical layers and the skin was closed (Vicryl 3-0, Ethicon, Issy Les Moulineaux, France). Animals received a bolus of saline (2 mL, subcutaneous) two to three times per day to replace the fluid lost during the surgical procedure until animals drunk alone. Rats were also kept under a heat lamp until thermoregulation was reestablished. A postoperative analgesic (buprenorphine, 0.03 mg·kg^{-1}, Bruprécare Multi-dose, Axience Santé Animale SAS, Pantin, France) was daily subcutaneously administered for 3 days. A wide spectrum antibiotic (Oxytetracycline, 400 mg·l^{-1}, Sigma Aldrich, Saint-Quentin Fallavier, France) was preventively given (in their drinking water) for 1 week. Manual bladder expression was performed at least twice daily. Postoperative nursing care also included administration of nutritional supplement (Nutri-Plus Gel, Virbac, Carros, France) for weight loss, visual inspection for skin irritation or decubitus ulcers, and cleansing the hindquarters with soap and water, followed by rapid drying of the fur with a bath towel.

Rats were maintained for 1 week until the second surgery, and they were not placed in an enriched environment to avoid environmental interference with the treatment.

2.6. Hydrogel Injection. Animals were reanesthetized with 3% isoflurane in oxygen, the skin and muscles were reopened, and the spinal cord at the injury site was exposed as described above. In the hydrogel groups, using a micropipette, 2 × 10 μL of sterile PNIPAAm-*g*-PEG copolymer was injected through the dura into the contused area. The gelation of the hydrogel occurred in situ after a few minutes. In the saline group, instead of hydrogel, saline was injected in the same amounts. Then, the muscles and skin were sutured (Vicryl 3-0, Ethicon) in anatomical layers. The animals received a bolus of saline (2 mL, subcutaneous) to replace the fluid lost during the surgical procedure two to three times per day until animals drunk alone, and they were kept under a heat lamp until thermoregulation was reestablished. Buprenorphine (0.03 mg·kg^{-1}, Axience Santé Animale S.A.S) was daily subcutaneously administered for 3 days to prevent pain. A wide spectrum antibiotic (oxytetracycline, 400 mg·L^{-1}, Sigma Aldrich) was preventively given in drinking water for 2 weeks. Manual bladder expression was performed at least twice daily until the bladder reflex was re-established (10−14 days postsurgery). Postoperative nursing care also included administration of nutritional supplement (Nutri-Plus Gel, Virbac) for weight loss, visual inspection for skin irritation or decubitus ulcers, and cleansing the hindquarters with soap and water, followed by rapid drying of the fur with a bath towel.

Rats in all groups were numbered randomly to ensure that the researchers were blind to the group and maintained the same for 9 weeks (W10) until the electrophysiological recordings. Animals were not placed in an enriched environment to avoid environmental interference with the treatment.

2.7. Endogenous Inflammation. Two weeks (W3, 3 weeks after the lesion) after the injection of the hydrogel or serum, an endogenous inflammatory reaction was evaluated at the lesion site. Animals were sacrificed with a lethal dose of anesthetic (Pentobarbital sodium, 390 mg/kg, i.p., Euthasol Vet., Dechra Veterinary Products S.A.S., Montigny-le-Bretonneux, France). A segment of spinal cord extending 5 mm rostral and caudal to the injury site was harvested, immediately immersed in isopentane, and stored at −80 °C until further analysis. A few days later, all samples were homogenized separately in 1 mL of PBS for 30 s with a handheld homogenizer (Ika Ultra Turrax disperser, Fisher Scientific SAS, Illkirch, France) equipped with plastic pestle tips that homogenize the tissue through vibrating motions. Then, the resulting mixtures were centrifuged (centrifuge Sigma 2-16 PK Centrifuge Fisher Scientific SAS, Illkirch, France) for 12 min (12,000*g*, 4 °C) and a fraction (50 μL) of the supernatant containing soluble proteins was used to evaluate inflammation. The concentrations of IL-1β, IL-6, and TNF-α were measured using enzyme-linked immunosorbent assay kits containing specific antibodies (RAB0272, RAB0311, and RAB0480, Sigma Aldrich, Saint-Quentin Fallavier, France) according to the instructions provided by the manufacturer, and all samples were run in duplicate. The absorbance was read at a wavelength of 450 nm using a microplate reader (Multiskan Microplate Photometer, Thermo Fisher Scientific, Life Technologies SAS, Courtaboeuf, France). Concentrations were determined from a standard curve and based on the amount of tissue weighed before homogenization. Thus, the interleukins levels were expressed as pg/g of spinal cord.

2.8. Behavioral Tests. Before surgery, the habituation period allowed to decrease the interindividual differences and to reach optimal performances. The week before surgery, the reference values (PRE-) of each test were recorded. Then, for 9 weeks (1 week postinjury, W1−W10), the tests were performed, once a week, by two experimenters blinded to the treatment group.

2.8.1. BBB-Test. The deficits and recovery of sensorimotor functions were assessed using the Basso−Beattie−Bresnahan test.[36] Briefly, animals were placed on an open-field environment made of a circular Plexiglas enclosure arena (95 cm

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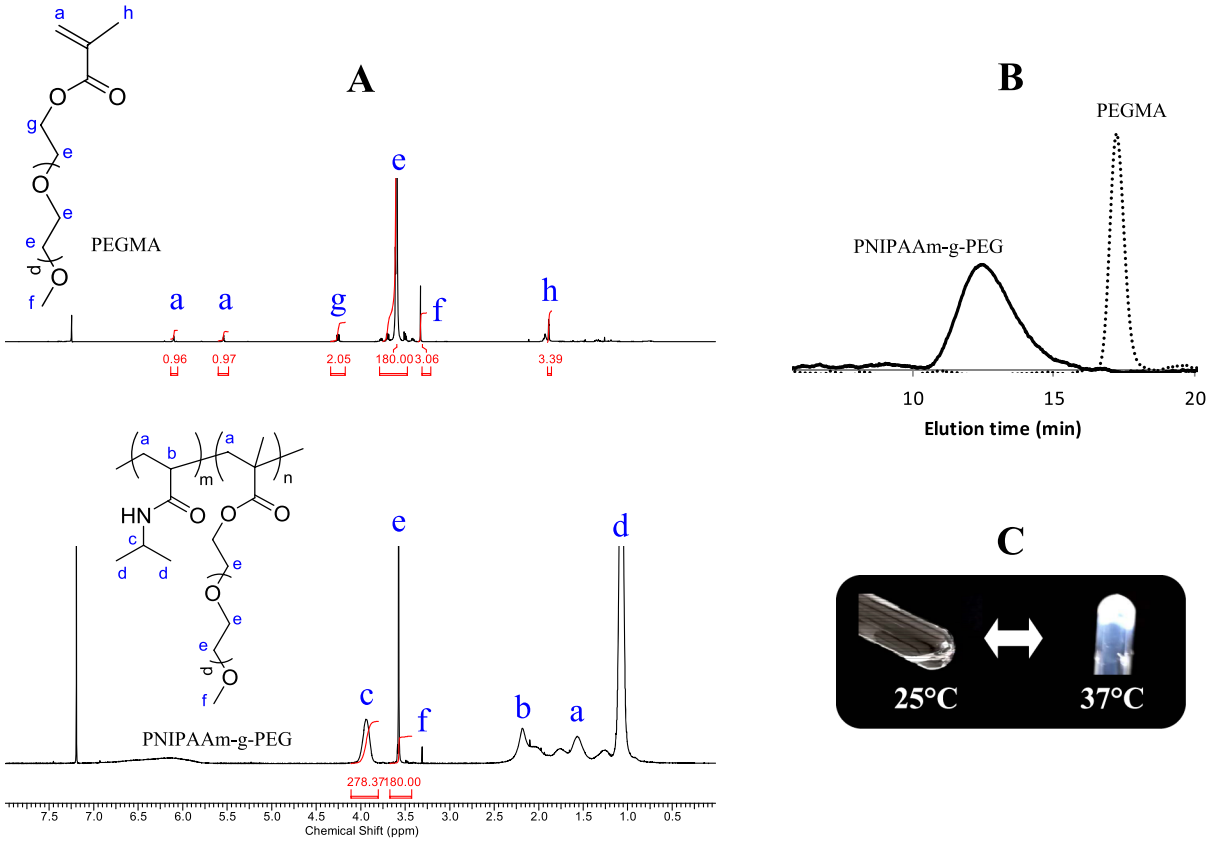

Figure 2. PNIPAAm-g-PEG preparation and characterization. (A) ^{1}H NMR (CDCl3) and (B) SEC (DMF) analyses of PEGMA and PNIPAAm-*g*-PEG copolymer. (C) Hydrogel formation upon heating to 37 °C (13.7 wt % in PBS, pH 7.4).

diameter and 40 cm wall height) with an antiskid floor. Once the rat walked continuously in the open field, the examiner conducted a 4 min testing session using the BBB locomotor rating scale based on 21 levels of locomotor behavior. A score of zero represents no hindlimb movement, while a score of 21 represents typical coordinated and stable rat walking. Animal movements were video-recorded during each session using a camcorder (MV 830i; Canon, Courbevoie, France) and analysis was carried out later on. For each rat, the locomotor scores for the hindlimbs were averaged together to yield one score per test session.

2.8.2. Ladder Climbing Test. Cortical control of fine sensorimotor coordination was tested while climbing an inclined ladder (10 cm × 150 cm) at a 45° angle. This task is an easily acquired spontaneous response that not requires compulsion or reward. As previously described,[37,38] this test was used to evaluate the sensorimotor capacities to correctly place the paw on round metal rungs (diameter: 0.4 cm spaced at equal intervals of 2 cm and at 150 mm high side walls) of a ladder while climbing. The rats were placed at the bottom rungs of the ladder and climbing was video-recorded with a camcorder (MV 830i; Canon) from a position below the ladder so that the ventral aspect of the animal was recorded. At the top of the ladder, the rats had access to a dark box. In contrast to unlesioned animals climbing readily, with all four paws locating and grasping the rungs without fault, lesioned animals showed varying degrees of difficulty in locating the ladder rungs with the affected legs. These later climbed the ladder using their forelimbs with the body weight support provided by the inclined position of the ladder. The video recordings were observed at slow-speed playback and the position of the hindpaws over the rungs was scored as follows: 0: the hindlimb was hanging in front of or behind the rungs and did not support climbing, 1: the hindlimb was used to support climbing but the hindpaw was not placed correctly on the rung, and 2: the hindpaw was correctly placed on the rung and the position was maintained while the trunk and the contralateral limb were moving up. The scores obtained on each side were averaged. Thus, a climbing score ranging from 0 (without success, i.e., 0 grip with the hindpaws) to 40 (animal climbed 40 rungs of the ladder without faults, i.e., 40 grips with the hindpaws, 20 per leg) was calculated and normalized to the maximal score. The mean ratio obtained at each session was expressed as percentage of the mean ratio obtained at week 0 (PRE-).

2.9. Treadmill Training. One week after injection (W2), animals from saline + E and PNIPAAm-*g*-PEG + E groups were enrolled in a treadmill training program performed 5 days/week and for 8 weeks (from W2 to W10). This program included walking on a motorized treadmill belt (Medical Développement, Saint-Etienne, France) for a daily session of 15 min at various speeds (from 15 to 30 m·min^{-1}) depending on the locomotor recovery level of each rat. Immediately after the SCI, rats were unable of autonomous hindlimb stepping and were therefore stimulated by pinching the perineum. This stimulation activated the CPG below the lesion and evoked, in most cases, a hindlimb locomotion (flexion/extension alternation) adapted to the belt speed but sometimes a hindlimb locomotion without plantar paw placement.[35] The trunk of the animal was manually maintained to limit the lateral imbalance as long as the animal needed a perineal stimulation to walk. When the animal was able to walk alone,

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the belt speed was increased in steps of 2 m·min^{-1} every 2 min as long as the animal supported the imposed speed within 30 m·min^{-1}.

2.10. Electrophysiological Recordings. Ten weeks postinjury (W10), animals were deeply anesthetized by intramuscular injection of a mixture containing ketamine (62.5 mg/kg^{-1}, 100 mg·m^{-1}, Ketamine 1000, Virbac, Carros, France) and xylazine (3.125 mg·kg^{-1}, 20 mg·mL^{-1}, Xilasyn2, Virbac) and prepared for electrophysiological recordings, as previously described.[39,40] Briefly, the peroneal nerves from both hindlimbs were dissected free from the surrounding tissues for stimulation. Then, the tibialis anterior muscles from both hindlimbs were exposed for electromyographic recording.

2.10.1. M- and H-waves. The *M*- and *H*-waves were recorded by stimulating the peroneal nerve. As previously described, the rate-dependent depression (RDD) of the *H*-reflex (i.e., the decrease in reflex magnitude relative to repetition rate) was analyzed by expressing the H_{max}/M_{max} ratio obtained at the stimulation frequencies of 1, 5, and 10 Hz to the H_{max}/M_{max} ratio obtained at a baseline frequency of 0.3 Hz.[16,39−41]

2.11. Euthanasia. According to ethical recommendations, at the end of the electrophysiological recordings, the animal was killed with an overdose of anesthetic (pentobarbital sodium, 390 mg/kg, i.p., Euthasol Vet.), and the spinal cord was removed to verify the extent of the lesion.

2.12. Statistical Analysis. Data were compared between all experimental groups using a software program (SigmaStat, San Jose, CA, USA). Normal data distribution was verified. A two-way analysis of variance (group factor × trials sessions) for repeated measures was used to compare the behavioral scores from all groups and over time and to compare H_{max}/M_{max} ratios from all groups and each stimulation frequency. Then, statistics were completed with a multiple comparison post-hoc test (Student−Newman−Keuls method). Data were expressed as mean ± standard error of the mean. The difference was considered significant when $p < 0.05$.

3. RESULTS

3.1. PNIPAAm-*g*-PEG Copolymer and Hydrogel Preparation. The PNIPAAm-*g*-PEG copolymer composed of 94/6 wt % in NIPAAm/PEG (determined from ^{1}H NMR integration, Figure 2A) and had a molecular weight of 83,000 g·mol^{-1} ($Đ$ = 2.1, from SEC, Figure 2B) modulated to afford both suitable LCST (33 °C determined by DLS, sufficiently below 37 °C) and sufficient chain entanglement. Nuclear magnetic resonance (NMR) and SEC analyses also showed that no residual (unreacted) PEGMA was present in the final copolymer (Figure 2A,B). The molecular weight of our copolymer was only slightly higher than the commonly reported renal cutoff (≈70 kDa),[42] enabling the copolymer to be potentially excreted through renal clearance. At 13.7 wt % in physiological solution (PBS, pH 7.4), hydrogel formation occurred instantaneously at 37 °C (Figure 2C). The storage modulus (G') and loss modulus (G'') of the copolymer hydrogel at 37 °C were in the range 25−50 and 17−20 kPa, respectively, typically matching with those of spinal cord.[43]

3.2. Animals. After SCI, all animals exhibited dramatic and bilateral hindlimb paralysis with no movement or only slight movements of the joint. On the 48 operated rats, 16 rats were sacrificed 2 weeks after saline (n = 8) or PNIPAAm-*g*-PEG (n = 8) injection for the evaluation of the endogenous inflammation, 2 rats died before the end of the experiments (1 at W8 in the saline + E group and 1 at W6 in the PNIPAAm-*g*-PEG + E group), and the others (n = 30) survived until the electrophysiological phase. All surviving animals underwent the weekly behavioral tests. Their weight did not drop throughout the experiment. During surgical preparation for electrophysiological recordings, in the saline group, one rat died during the surgery because of respiratory failure.

3.3. Endogenous Inflammation. Two weeks following injection (W3), measurement of IL-1β, IL-6, and TNF-α levels at the lesion site did not show difference between the saline and PNIPAAm-*g*-PEG groups, indicating that the hydrogel did not produce additional inflammatory reaction (Figure 3).

3.4. Behavioral Tests. *3.4.1. BBB Test.* Analysis of the BBB scores showed that the scores significantly ($p < 0.001$) dropped 1 week (W1) postinjury in all lesioned groups compared to preinjury values (PRE-) and then increased slowly during the following 9 weeks reaching at W10 a score of

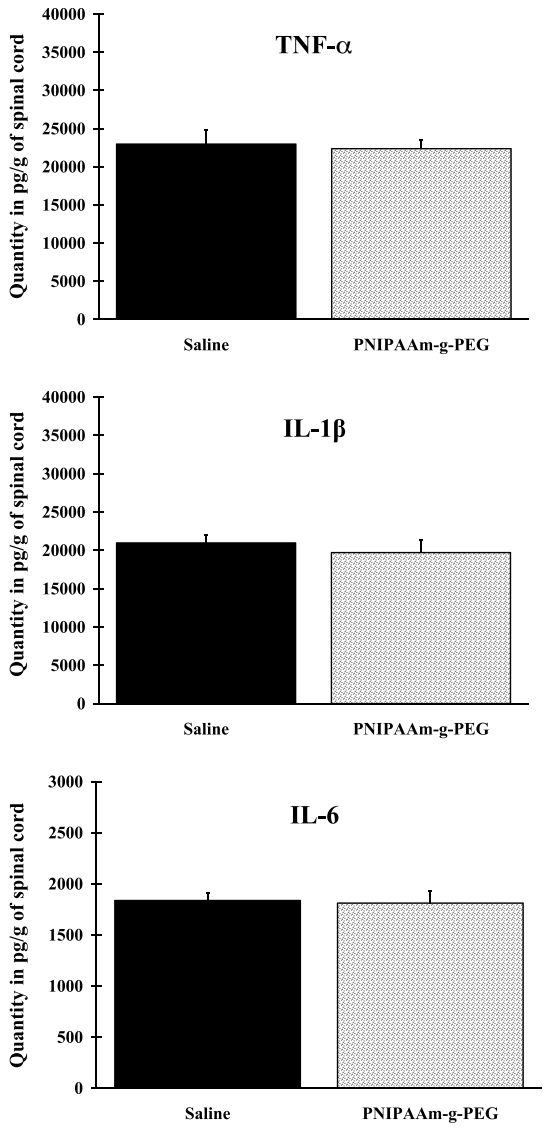

Figure 3. Inflammatory reaction at the lesion site. Comparison of IL-1β, IL-6, and TNF-α levels at the site of injury in the saline and PNIPAAm-*g*-PEG groups, 2 week postinjection (W3, 3 weeks postinjury), does not reveal any additional inflammation when the PNIPAAm-*g*-PEG copolymer is added.

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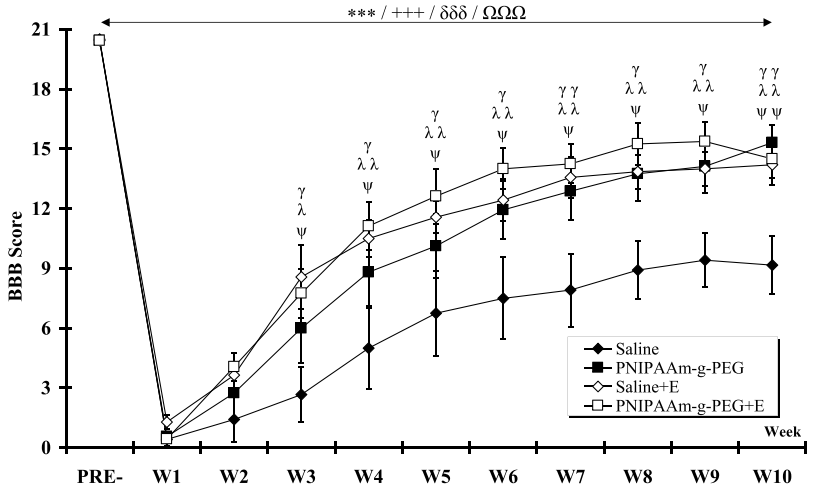

Figure 4. BBB-derived locomotor rating scale. After the SCI, the BBB score in each group drops significantly, and then a slow recovery is observed until W10. From W3, the BBB score in the saline group remains lower than in the three other groups. Significant difference in the BBB scores is indicated by a *(saline group, PRE-vs postinjury), + (PNIPAAm-*g*-PEG group, PRE-vs postinjury), δ(saline + E group, PRE-vs. postinjury), Ω(PNIPAAm-*g*-PEG + E group, PRE-vs postinjury), λ(saline group vs. PNIPAAm-*g*-PEG + E group), γ(saline group vs saline + E group), and ψ(saline group vs PNIPAAm-*g*-PEG group). (1 symbol, $p < 0.05$, 2 symbol, $p < 0.001$, and 3 symbols, $p < 0.001$).

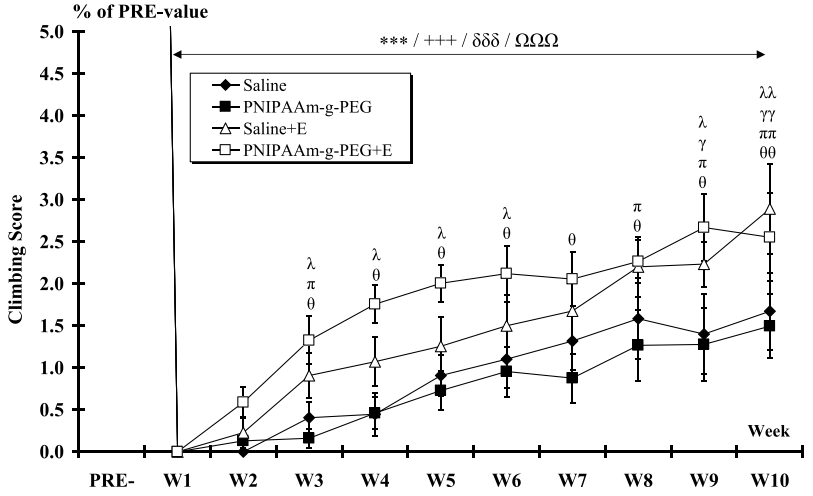

Figure 5. Ladder climbing test. After the SCI, the climbing score in each group drops significantly, and then a slow recovery is observed until W10. From W3, some differences are observed between groups. Significant difference in the climbing scores is indicated by a* (saline group, PRE-vs postinjury), + (PNIPAAm-*g*-PEG group, PRE-vs postinjury), δ(saline + E group, PRE-vs postinjury), Ω(PNIPAAm-*g*-PEG + E group, PRE-vs postinjury), λ(saline group vs PNIPAAm-*g*-PEG + E group), γ(saline group vs saline + E group), θ(PNIPAAm-*g*-PEG group vs PNIPAAMm-*g*-PEG + E group), and π(PNIPAAm-*g*-PEG group vs saline + E group). (1 Symbol, $p < 0.05$, 2 symbol, $p < 0.001$, and 3 symbols, $p < 0.001$).

9.1 ± 1.4 (intermediate stage: intervals of uncoordinated stepping) in the saline group and above 14 (late stage: consistent forelimb and hindlimb coordination with consistent weight support) for the others groups (PNIPAAm-*g*-PEG: 15.3 ± 0.8; saline + E: 14.2 ± 1.0; and PNIPAAm-*g*-PEG + E: 15.1 ± 2.3). Significant differences between the saline group and others groups were detected at the beginning of W3 following injury. No difference was found between the saline + E, PNIPAAm-*g*-PEG, and PNIPAAm-*g*-PEG + E groups (Figure 4).

3.4.2. Ladder Climbing Test. Two weeks (W2) after the lesion, the climbing scores of each group dropped significantly ($p < 0.001$). Then, a recovery was observed from W1 to W10 in all groups (Figure 5). Furthermore, the results indicated that the exercised groups recovered more quickly, reaching, at W10, higher ($p < 0.01$) scores than in the nonexercised groups. However, despite a recovery, at W10, the score of each group remained below the maximum score that could be achieved.

3.5. Electrophysiological Recordings. The values of the H_{max}/M_{max} ratios measured at the baseline stimulation (0.3 Hz) were 0.38 ± 0.08, 0.39 ± 0.04, 0.38 ± 0.05, and 0.27 ± 0.03 for saline, PNIPAAm-*g*-PEG, saline + E, and PNIPAAm-*g*-PEG + E groups, respectively. Although the ratio in the PNIPAAm-*g*-PEG group seemed to be lower, statistical analysis did not reveal significant difference between groups. Furthermore, except for saline and PNIPAAm-*g*-PEG + E, H_{max}/M_{max} ratios decreased when the frequency of stimulation was increased (Figure 6). Indeed, in the saline + E group, the ratio values at 1, 5, and 10 Hz were 94.02 ± 3.36, 93.44 ± 3.68, and 88.88 ± 3.92% of the ratio measured at the baseline stimulation, respectively. In the PNIPAAm-*g*-PEG group, the depression to stimulation was higher than in the saline + E group, and the ratio values at 1, 5, and 10 Hz were 95.62 ±

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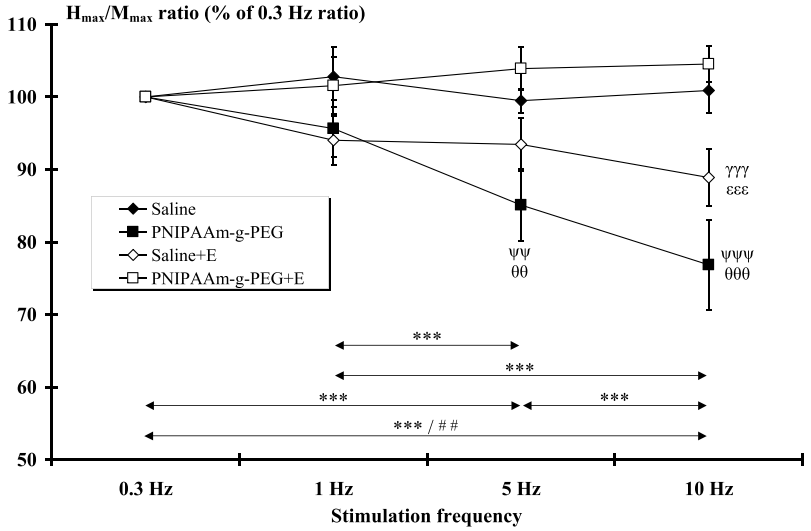

Figure 6. *H*-reflex recordings. *H*-reflex sensitivity, measured after increasing the frequency of stimulation, shows a depression in the saline + E and PNIPAAm-*g*-PEG + E groups. In the PNIPAAm-*g*-PEG and saline + E group, the significant difference between the H_{max}/M_{max} ratio is indicated by a* and a#, respectively. For a given frequency, the significant difference in the H_{max}/M_{max} ratio is indicated by a ψ (PNIPAAm-*g*-PEG group vs saline group), θ(PNIPAAm-*g*-PEG group vs PNIPAAm-*g*-PEG + E group), γ(saline + E group vs saline group), and ε(saline + E group vs PNIPAAm-*g*-PEG + E group). (2 Symbols, $p < 0.01$, and 3 symbols, $p < 0.001$).

3.91, 85.10 ± 4.91, and 76.84 ± 6.16% of the ratio measured at 0.3 Hz, respectively. Statistical analysis indicated that the value of the ratio decreased significantly ($p < 0.01$) at 10 Hz in the saline + E group compared to the ratio measured at 0.3 Hz. In the PNIPAAm-*g*-PEG group, the value of the ratio was significantly lower at 5 Hz ($p < 0.001$) and 10 Hz ($p < 0.001$) compared to that measured at 0.3 and 1 Hz. Comparison between groups indicated a significant ($p < 0.01$) difference between the PNIPAAm-*g*-PEG group and saline group at 5 Hz. At this frequency, a significant ($p < 0.01$) difference was also observed between the PNIPAAm-*g*-PEG group and the PNIPAAm-*g*-PEG + E group. At a frequency of 10 Hz, a significant ($p < 0.001$) difference was observed between the PNIPAAm-*g*-PEG group and the saline group. Furthermore, a significant difference was also observed between the PNIPAAm-*g*-PEG group and the PNIPAAm-*g*-PEG + E group ($p < 0.001$), between the saline + E group and the saline group ($p < 0.01$), and between the saline + E group and the PNIPAAm-*g*-PEG + E group ($p < 0.01$).

4. DISCUSSION

Because the translational potential of novel treatments should be investigated in SCI contusion models, a T10 contusion was induced by a weight-drop leading to severe sensorimotor deficits at a chronic stage. Then, a thermosensitive and thermoreversible physically cross-linked poly(*N*-isopropylacrylamide)-poly(ethylene glycol) hydrogel was injected into the lesion area 1 week (W1) after the contusion, and recovery was evaluated for 9 weeks postinjury (from W1 to W10). In addition, to improve recovery, animals were enrolled in a treadmill training program performed 5 days/week, for 8 weeks with sessions of 15 min at speed varying from 15 to 30 m·min^{-1}.

Measurement of the level of proinflammatory cytokines indicates that the hydrogel did not induce additional inflammatory reaction 2 weeks postinjection (W3, 3 weeks post-lesion) within the lesion area. Indeed, the mean levels of IL-1, IL-6, and TNF-α were similar in the saline and PNIPAAm-*g*-PEG groups.

The BBB and ladder climbing scores were better in PNIPAAm-*g*-PEG and exercised (saline + E and PNIPAAm-*g*-PEG + E) groups, indicating the beneficial effects of the hydrogel and the exercise, even when they were combined.

Finally, recording of the *H*-reflex depression to various frequencies of stimulation indicated an improvement in the PNIPAAm-*g*-PEG and in the saline + E groups, but when the two strategies were combined, no improvement was observed.

4.1. PNIPAAm-*g*-PEG Does Not Increase the Inflammatory Reaction. A spinal cord contusion leads to an inflammatory reaction at the lesion site with the infiltration of leukocytes and activation of glial cells which can be, for some, neurotoxic and, for others, neuroprotective.[44−48] The inflammatory reaction starts within the 1 h after injury and remains several weeks with a peak of astrocytes and macrophages 14 days after the injury.[49] Inflammation is a physiological process that removes the damaged tissue and initiates the healing process. If it persists and if it is overactivated, the inflammatory reaction becomes devastating and limits regeneration. Thus, because it was described that biomaterials chemically and physically interact with the immune cells and may activate macrophages,[50] we verified that our physically cross-linked PNIPAAm-*g*-PEG hydrogel did not trigger an additional inflammatory reaction 2 weeks after its injection into the lesion site. Calculation of the mean level of three proinflammatory cytokines (Il-1β, Il-6, and TNF-α) confirmed that our hydrogel did not increase the inflammation in the contused spinal cord, suggesting that it exhibits mandatory characteristics to be used as a CNS scaffold.

4.2. PNIPAAm-*g*-PEG and Exercise Improve Sensory and Motor Recovery. Although a spontaneous recovery of locomotor function due to neuroplasticity was observed over time in all groups after the spinal cord contusion as previously described,[48,51−53] groups receiving only the hydrogel or only daily training or receiving the hydrogel and training presented the higher BBB scores and reached a score above 14 in which a consistent forelimb and hindlimb coordination with consistent

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weight support was observed. However, our results failed to show an addition of the effects when the two strategies were combined because the scores reached in the group where hydrogel was combined to the exercise were similar. Recently, Tom et al.[26] did not report a further improvement in a model of moderated T9 spinal cord contusion (NYU impactor device with a 10 g, 2 mm-diameter rod head dropped from a height of 25 mm) after injection of a chemically cross-linked PNIPAAm-*g*-PEG hydrogel loaded with BDNF/NT3 or uninjected lesioned animals trained for up to 8 weeks with the body-weight-supported treadmill training (BWSTT, 75% BWS at 7 $cm{\cdot}s^{-1}$ speed for 5 days a week, 1000 steps/days)[54] compared to untrained animals. Previously, Singh et al.[54] also concluded that such training program did not induce a higher BBB score after the same spinal contusion compared to untrained animals. The authors concluded that their contusion injury model preserved most of the ventral and ventral−lateral descending pathways[55] that led to postinjury locomotor recovery in both trained and untrained animals with no additional effect of the training program. However, the authors showed better kinematic parameters (swing duration, step height, and length) in trained compared to untrained rats.

One hypothesis to explain the discrepancies between our results and previous results is the difference in the extent of the lesion and the training method. In our case, the lesion was a severe contusion (NYU impactor device with a 10 g, 2.5 mm diameter rod head dropped from a height of 50 mm) and the continued treadmill training was performed at a high speed (15−30 $m{\cdot}m^{-1}$) by sustaining manually the animals and by stimulating the locomotion with perineum pinches. This step training on the treadmill was previously compared to passive bike-training performed on motorized apparatus.[56] These two types of exercise increase the NT-3, NT-4, and BDNF protein levels.[56] Furthermore, while the two training modes were described to increase functional recovery, only step-training method was described to increase the glial cell-derived neurotrophic factor (GDNF) levels,[56] which is known to promote locomotor recovery.[57−59] Other studies confirmed an increase of the locomotor outcomes,[9,60] of the expression of growth-associated protein-43 (GAP-43) at the site of SCI, and of the number of neurons expressing tyrosine hydroxylase in the spinal cord segment below the lesion.[60] In addition, the intensive strengthening of the spared pathways of the contusive spinal cord could also make a difference between bike-training and step-training. Indeed, as previously suggested, the strengthening of some spared supraspinal fibers seems to be the key to locomotor recovery after training.[54,61] Thus, we cannot exclude any contribution of the perineal stimulation; the afferent feedback induced by perineal stimulation could potentiate the effect of training on spinal networks such as CPG and/or strengthening the spared pathways through the increase of GDNF levels and plasticity.

Concerning, the ladder climbing test, results indicated that only group (saline + E and PNIPAAm-*g*-PEG + E) performing a daily exercise for 8 weeks presented higher scores than untrained groups (saline and PNIPAAm-*g*-PEG). However, despite a slight recovery, the scores achieved were very low (around 3% of the maximal score). The ladder climbing test is a test based on sensorimotor integration to correctly grip a fixed rung while the animal climbed up an inclined ladder.[37] It is a complex foot fault test employed to assess sensory (afferent inputs) and motor (efferent inputs) deficits and incoordination after SCI.[62] The ladder climbing test is more sensitive than the BBB test because it intends simultaneously test tactile sense, proprioception, and motor performances. Thus, we can hypothesize that the few fine recovery induced by training can only be recorded with a sensitive test such as the ladder climbing test and that the recovery induced by the PNIPAAm-*g*-PEG copolymer alone did not concern fine sensorimotor coordination.

4.3. PNIPAAm-*g*-PEG and Exercise Restore the RDD of the *H*-reflex. Our results suggest that the PNIPAAm-*g*-PEG hydrogel (PNIPAAm-*g*-PEG group) could provide a suitable environment that could induce beneficial changes allowing recovery of the transmission between the Ia afferent fibers and α-motoneurons and supralesional inhibition from spared or regenerating descending pathways. To a lesser extent, the same results were observed in the group of animals that exercised daily (saline + E group), namely, a *H*-reflex depression when the stimulation frequency was increased, suggesting that step-training activated mechanisms leading to post-lesional adaptive plasticity. Finally, when the hydrogel was associated with exercise (PNIPAAm-*g*-PEG + E group), the RDD remained lost as for untreated animals (saline group), suggesting that the beneficial effect of both strategies is cancelled out, that is, the mechanisms initiated by hydrogel are certainly not consolidated when the mechanisms initiated by the exercise begin.

Spasticity is frequently observed after a SCI. It results in descending pathway interruption and disorganization of spinal networks. Hyperreflexia is the most studied component of spasticity that may result, among other things, in the decrease of presynaptic inhibition of Ia afferents, changes in α-motoneuron excitability (persistent inward current), and/or changes in synaptic transmission (release of neurotransmitter, postsynaptic receptor, number of synapses, etc.) in the reflex arc.[63−65] More recently, a correlation between serotonin immunoreactivity, 5-HT_{2A} receptors, and potassium chloride cotransporter (KCC2) expression with enhancement of the monosynaptic reflex after a spinal cord contusion was also reported.[66−69] Thus, in order to evaluate this component of spasticity, we recorded the *H*-reflex and measured the H_{max}/M_{max} ratio under different frequencies of stimulation. In the absence of SCI, the H_{max}/M_{max} ratio decreases when the frequency of stimulation increases. However, after a SCI, an attenuation of the RDD was shown.[70]

Our results indicated that the RDD of the *H*-reflex was abolished in the saline group confirming previous results using section[16,39,40,64,71] or contusion[26,69] rat models of SCI. Furthermore, our results showed a decrease in the H_{max}/M_{max} ratio in the PNIPAAm-*g*-PEG group, indicating the beneficial effect of the hydrogel when delayed injected in a contusive spinal cord. As previously described, transplanting a biomaterial into a spinal lesion cavity immediately after a lesion may limit the cascade of events of the secondary injury and the development of the glial scar, which is a physical barrier preventing the axonal regrowth.[16,17,39] In a recent study, Tom et al.,[26] using a chemically cross-linked (use of dimethacrylate as a cross-linker) PNIPAAm-*g*-PEG hydrogel loaded with BDNF/NT3 and injected into the injured area 1 week after a moderate T9/T10 spinal cord contusion, reported a loss of the RDD and no difference with lesioned untreated animals. Here, we reported a restoration of the RDD after a T10 severe contusion and the use of physically noncovalent cross-linked PNIPAAm-*g*-PEG hydrogel. It is possible that the cross-linker used in the study of Tom et al.[26] was not beneficial and

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prevented the RDD recovery even when combined to neurotrophins.

In addition, we found an attenuated RDD in the saline + E group, indicating the beneficial effect of the exercise. It was previously reported that a BWSTT for up 8 weeks induced a restoration of the RDD in animal with a thoracic contusion injury.[54] Similar results were obtained with 1 month (2 × 30 min bouts of cycling with a 10 min rest, 5 days/week), 1.5 months (1 h/day, 5 days/week), 3 months (1 h/day, 5 days/week), or 4 months (15 min/day, 5 days/week) of passive motorized bicycle exercise training started immediately or with a delay of 30 days after a spinal cord transection in which the onset of hyperreflexia is after the seventh day post-injury.[56,63,64,72−75] It was observed that the effect of the exercise persisted after the end of the training protocol,[75] and it was concluded that the RDD recovery was correlated with an increase in neurotrophic factor proteins (BDNF, NT-3, and NT-4)[56,76−78] and an increase of BDNF upregulating KCC2 expression and restoring RDD after SCI.[79] However, it was noted that only step-training generated by spinal networks triggered by afferent feedback increased GDNF levels.[56] Finally, it was noted that such mode of training restored the RDD, decreased the *H*-reflex threshold, and facilitated the recruitment of the motoneuronal pool in response to afferent input.[56]

In our experiments, the step-training on the treadmill was associated with perineal stimulation and manual assistance.[56,80] As previously described by numerous authors, we noted behavioral recovery and a RDD restoration in trained animals compared to untrained animal highlighting that rehabilitation based on repetitive and rhythmical movements that involve alternation between flexion and extension of the hip, knee, and ankle provides sensorimotor information to activate the spinal networks necessary to allow functional recovery.[81−83] Compared to passive movements induced by a motorized apparatus, step-training is a complex task involving weight-bearing, foot placement, and constant control of posture and position of limb joints.[56]

Finally, our results did not show additive benefit when PNIPAAm-*g*-PEG was combined with exercise. Indeed, in the PNIPAAm-*g*-PEG + E group, the RDD was abolished as for the saline group. It is difficult to find an explanation for this cancellation of the effect of hydrogel or exercise when the two strategies are combined, especially because the BBB test showed improved scores for this combination compared to animals receiving only a saline injection. Tom et al.[26] reported a restoration of the RDD but lower BBB scores than lesion alone with a combinational strategy (chemically cross-linked hydrogel + BDNF/NT-3 + exercise). The authors also reported the same results when PNIPAAm-*g*-PEG was loaded with BDNF/NT-3 but did not evaluate the effect of the hydrogel alone or the effect of the neurotrophins alone, suggesting that the effect observed could be due to the neurotrophic factors. Because it was described that some hydrogels could modulate the inflammatory response by attenuating the M1 inflammatory macrophage and by promoting the polarization of M2 anti-inflammatory macrophages,[84−86] we cannot not exclude that the PNIPAAm-*g*-PEG hydrogel could induce biochemical reactions incompatible with those induced by exercise; that is, the addition of the beneficial effect of the hydrogel to the beneficial effect of the exercise leads to deleterious effects.

5. CONCLUSIONS

In this study, we showed that the PNIPAAm-*g*-PEG hydrogel copolymer and step-training exercise exhibit mandatory properties to be used as a CNS scaffold after a thoracic spinal cord contusion. Indeed, animals receiving the hydrogel (PNIPAAm-*g*-PEG group) 1 week after the lesion or postinjury enrolled for 8 weeks in an exercise training program (saline + E group) showed significant locomotor recovery. Such improvement was also observed in animals simultaneously treated with the hydrogel and performing the daily step-training exercise (PNIPAAm-*g*-PEG + E group). However, the combination of the two strategies did not show better results compared with each strategy separately performed. At the level of the sensorimotor loop, the PNIPAAm-*g*-PEG hydrogel and exercise induce beneficial changes but not when they were combined. It would be interesting to delay the start of training or use another less intense type to verify if its effects can be potentiated to those of hydrogel.

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Author Contributions

M.B. and O.A. are contributing authors. All the authors equally conceived, designed, and performed the experiment; analyzed the data; and performed the statistical analysis and wrote the paper. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

All data analyzed during this study are included in this publication. The data sets analyzed during the current study are available from the corresponding author on reasonable request.

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